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Phenotypic, Genetic, and Cytogenetic Evidence of Hybridization Between Species of Trans-Andean Tamarins (Genus *Saguinus*)

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Abstract Incomplete reproductive isolation and hybridization is relatively frequent in primates. However, no cases of hybridization between formally recognized species have been reported in tamarins (genus *Saguinus*), a highly specious group of Neotropical primates. Here, we provide evidence from different sources to demonstrate three cases of hybridization in captivity between species of *Saguinus* distributed west of the Andes (trans-Andean). To do this, we described fur color patterns, genotyped 12 microsatellite loci, sequenced the mitochondrial hypervariable region I, and generated chromosomal R bands for the three formally recognized species and the new hybrids of trans-Andean tamarins. We identified one case of interbreeding between the white-footed tamarin (*Saguinus leucopus*) and the cotton-top tamarin (*S. œdipus*) and two independent reciprocal crosses

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of *S. leucopus* and the Geoffroy's tamarin (*S. geoffroyi*). All these hybrids exhibit intermediate phenotypes between parental species, and genetic data are consistent with first-generation hybridization. Cytogenetic data suggest that the *S. leucopus* × *S. œdipus* hybrid is sterile, as it is a female with XY karyotype apparently affected by a condition known as gonadal dysgenesis. Trans-Andean tamarin species occur in northwest Colombia with parapatric distributions bounded by major rivers. Potential contact zones, either natural or anthropogenic, might facilitate hybridization in the wild, but this scenario remains to be assessed. Our findings warrant future studies focused on the evolutionary mechanisms of reproductive isolation in tamarins. Given the risk of hybridization, caution should be taken in management and conservation of tamarins.

Keywords Chimerism · Microsatellite · Mitochondrial DNA · Gonadal dysgenesis · Hybrid · Neotropical primate

Introduction

Hybridization between closely related species with sympatric or parapatric distributions occasionally occurs in contact zones, but human intervention in habitats and populations may also facilitate contact and interbreeding between formerly isolated taxa (Banes *et al.* 2016; Detwiler *et al.* 2005; Dias *et al.* 2013; Ruiz-Miranda *et al.* 2006). Documenting these events is very important to understand how biological mechanisms of reproductive isolation and speciation evolve. Identifying incomplete reproductive barriers between closely related taxa may also provide valuable information to anticipate the risk of unintended interbreeding between distinct species in *ex situ* populations or as a consequence of population management and intervention in the wild.

Reproductive isolation is pivotal in the definition of the widely used Biological Species Concept (Mayr 1942). However, the frequent violation of this principle required a reappraisal of this concept in the study of primates (Frankham *et al.* 2012; Groves 2004, 2012). Hybridization between recognized species of Old World primates has been shown through direct evidence or demonstrated with genetic and phylogenetic data for *Cercopithecus*, *Colobus*, *Eulemur*, *Homo*, *Hylobates*, *Macaca*, *Microcebus*, *Nomascus*, *Papio*, *Pongo*, *Propithecus*, *Symphalangus*, *Theropithecus*, and *Trachypithecus* (Arnold and Meyer 2006; Detwiler *et al.* 2005; Gligor *et al.* 2009; Hirai *et al.* 2007; Myers and Shafer 1979; Rosenblum *et al.* 1997; Roos *et al.* 2011; Sankararaman *et al.* 2012; Satkoski Trask *et al.* 2013; Tosi *et al.* 2000; Xu and Arnason 1996). In contrast, only a few cases of interspecific hybridization in the New World have been described for *Saimiri*, *Alouatta*, *Cebuella*, *Cebus*, and *Callithrix* (Agostini *et al.* 2008; Aguiar *et al.* 2008; Cortés-Ortiz *et al.* 2007; Malukiewicz *et al.* 2015; Mendes 1997; Neusser *et al.* 2004; Nieves *et al.* 2008; Silva *et al.* 1992).

The genus *Saguinus* (tamarins; family Callitrichidae) is one of the most specious and widely distributed primate genera in the New World (Hershkovitz 1977; Rylands and Mittermeier 2013). However, hybridization has been described only between subspecies of the Amazonian saddleback tamarin (*Saguinus fuscicollis*), recently reclassified as *Leontocebus fuscicollis* (Cheverud *et al.* 1993; Peres *et al.* 1996; Rylands *et al.* 2016). The *œdipus* group, also known as the trans-Andean tamarins given their distribution west of the Andes, is a monophyletic clade comprising the species *Saguinus œdipus*, *S. leucopus*,

and *S. geoffroyi* (Groves 2001). This group diverged from Amazonian tamarins around 4.9 million years ago (Ma) and started diversifying around 3.1 Ma (Buckner *et al.* 2015; Defler 2010). However, despite their parapatric distribution in northwestern South America and relatively recent diversification, no hybrids have been formally described.

Here, we identified three independent cases of interbreeding between trans-Andean tamarin species in captivity based on fur color patterns, as well as molecular and cytogenetic data that imply recent hybridization. We discuss the evolutionary implications of this evidence, the potential for hybridization in the wild, and the consequences for management and conservation of these species.

Methods

Hybrid Specimens

We detected three unrelated cases of potential interbreeding between tamarin species in wildlife shelters between 2015 and 2017, which we named after the wildlife shelter where they were held (CVC, AMVA, and CPN [1 and 2]). The first case is a captive-born female reported at the Animal Refuge of Corporación Autónoma del Valle del Cauca (CVC) in Palmira (Valle del Cauca Department) in west Colombia (Fig. 1). This female's *Saguinus leucopus* mother originally shared the same enclosure with a conspecific male, but owing to recurrent aggression from the female and the limited area available, they were split up and the female was moved to another enclosure and housed with a male *S. geoffroyi* in March 2014. The female conceived before the death of the male in November from an infected wound. She gave birth to twins in December 2014, but only one of the twins (a female) survived to adulthood.

The second case is a female tamarin with unique fur color pattern, seized from illegal holders in May 2015, and moved to the Animal Refuge of Área Metropolitana del Valle de Aburrá (AMVA) in Barbosa (Antioquia Department) in northwest Colombia. Owing to its geographic location and comparatively large facilities, this center receives fauna rescued from illegal traffic and possession in several departments of northwest Colombia. According to their own records, all the tamarin specimens held at AMVA between 2010 and 2014 were trans-Andean species (*Saguinus œdipus* 49%, *S. leucopus* 44%, and *S. geoffroyi* 7%) and none were Amazonian tamarins.

The third case involves female twins born in captivity at the Animal Refuge of Corporación Autónoma Regional de la Frontera Nororiental (CPN) located in El Zulia (Norte de Santander Department) in northeast Colombia. They are the offspring of a male *Saguinus leucopus* and a female *S. geoffroyi* that were originally housed together in December 2013 owing to constraints in area availability. They mated and the female *S. geoffroyi* gave birth to the hybrid female twins in April 2014 (CPN1 and CPN2).

Sample Collection

Medical staff weighed and sedated each specimen following domestic protocols, and measured head, body, tail, and limbs (body mass; body mass index; and length of body, head–body, tail, thigh, foot, forearm upper arm, heard breath, and ear (Electronic Supplementary Material [ESM] Table SI; see Soto-Calderón *et al.* (2016) for details). They

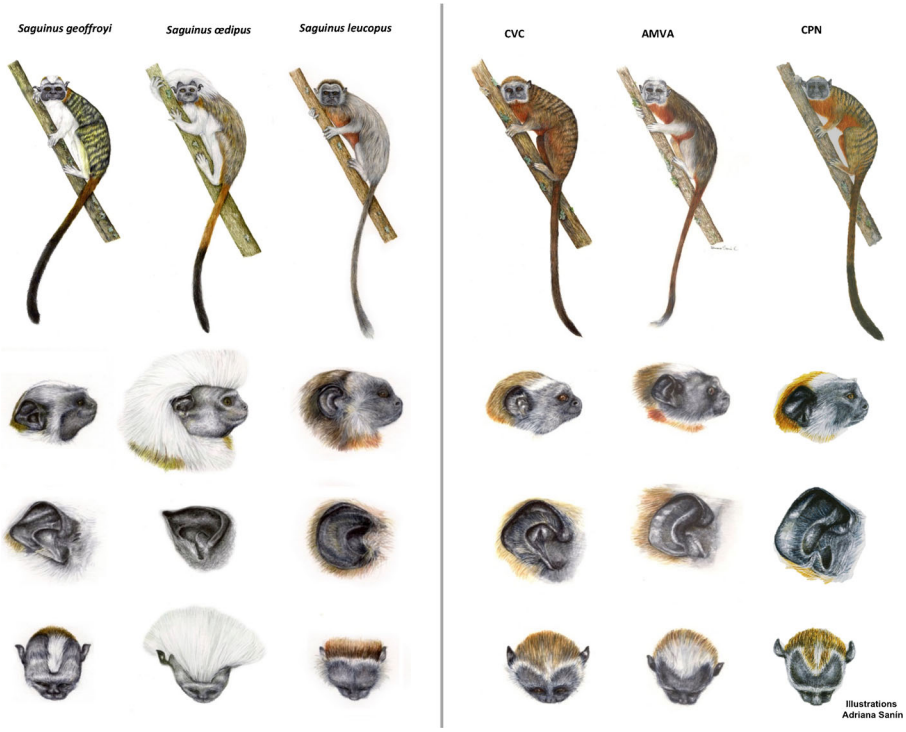

Fig. 1 Illustrations of the color pattern, right-side profile, right ear and crown of the three trans-Andean species of *Saguinus* (left) and three hybrid specimens (right). We detected the hybrids between 2015 and 2017 in three different animal refuges in west (CVC), northwest (AMVA) and northeast (CPN) Colombia. Illustrations by Adriana M. Sanín.

checked that all the specimens were adults and females exhibited no symptoms of pregnancy. They collected 1.5–2.0 ml of blood from the femoral vein (<1% of body mass) and split it in two Minicollect Tubes® (Monroe, NC, USA), one containing EDTA for genetic analysis and the other containing heparin for cytogenetic analyses. They administered a subcutaneous volume of saline solution equivalent to the amount of collected blood to each individual.

In addition to vertical cell and gene transmission from parents, horizontal cell transfer (mainly hematopoietic cells) from a twin during embryonic development is relatively frequent in callitrichid monkeys (marmosets and tamarins) (Ross *et al.* 2007; Sweeney *et al.* 2012). This phenomenon, known as cell chimerism, may result in the presence of three or even four alleles per autosomal locus in a given specimen and complicate genetic analyses (Ross *et al.* 2007; Sweeney *et al.* 2012). In those specimens of the parental species or hybrids in which we detected this signature of chimerism in at least 1 out of 12 autosomal loci, we also gathered data from hair DNA. Although the amount of DNA from hair follicles is substantially lower than that from blood, it is a non-hematopoietic tissue relatively less affected by chimerism than other tissues (Sweeney *et al.* 2012). Therefore, we also plucked between 20 and 30 hair roots from the abdominal region of each individual and stored them in a sterile dry tube.

We also collected blood and hair samples of *Saguinus geoffroyi*, *S. leucopus*, and *S. œdipus*, which we used along with body measurements and proportions as a

reference to investigate admixture levels of the four candidate hybrids. All the samples of *S. leucopus* were previously obtained from wild-caught georeferenced individuals across the geographical species range (Defler 2010). Samples of the two other species were taken from rescued specimens held in animal refuges. We cannot guarantee that these specimens are free of interspecies hybridization in previous generations. However, they were healthy specimens that exhibited the characteristic fur and color patterns of the three species, with no externals signs of intermediate or atypical variations.

DNA Extraction

We extracted genomic DNA from 200 μl of blood following a standard salting-out protocol (Sambrook *et al.* 1989). We also extracted DNA from 50 to 100 μl of blood or from *ca*. 30 hair bulbs using the DNAeasy Blood and Tissue kit (Qiagen, Hilden, Germany). We quantified DNA concentration using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Autosomal Genetic Data

To identify the parental species and level of genetic admixture of the candidate hybrids, we tested the specificity and polymorphism of 12 autosomal microsatellite loci originally designed for *Saguinus bicolor* and *Callithrix jacchus*, and optimized their amplification for trans-Andean tamarins (Böhle and Zischler 2002; Nievergelt *et al.* 2000). We generated data for the 4 hybrids, 8 *S. geoffroyi*, 19 *S. leucopus*, and 19 *S. œdipus* specimens. We labeled forward primers with a distinctive fluorochrome as shown in Table I. Amplification reactions contained 20–50 ng of genomic DNA extracted from blood, 1× KCl buffer, 1.5–2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, and 0.5 U of *Taq* polymerase in a final volume of 15 μl (Fermentas, Waltham, MA, USA). We amplified these loci at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, annealing temperature (see Table I) for 45 s and 72 °C for 1 min, followed by a final step of 72 °C for 10 min. We combined polymerase chain reaction (PCR) products in four three-locus multiplexes and ran them in an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). We visually inspected raw microsatellite data, edited them with Geneious v.6.1.5 (Biomatters Ltd.), and assigned alleles to a specific bin with the program Flexi Bin (Amos *et al.* 2007). We measured polymorphism levels as the observed number of alleles (*A*) and the effective number of alleles (n_e). Finally, we tested for significant departure of genotype frequencies from the Hardy–Weinberg (HW) equilibrium with a 1-million step Markov chain as implemented in Arlequin v3.5.1.2 (Excoffier and Lischer 2010).

To test the power of the microsatellite data to recognize differences among the three *Saguinus* species and estimate ancestry levels in the candidate hybrids, we used a Bayesian assignment test in the program Structure v2.3.4 (Pritchard *et al.* 2000) with three *a priori* clusters ($K = 3$). To do this, we ran 10 iterations of a Markov chain Monte Carlo (MCMC) with 50,000 steps and a 10% burn-in, using the default parameters of admixture ancestry and correlated allele frequencies. We estimated the contribution of each tamarin species to the genome of each hybrid from the mean of ancestry coefficients estimated in 10 independent iterations (95% confidence interval). We also used NewHybrids (Anderson and Thompson 2002) to estimate the probabilities that hybrids were assigned to one of six classes (species 1, species 2, F1, F2, backcross to

Table I Primers and amplification conditions used to genotype 12 microsatellite loci in trans-Andean tamarins

Locus	Primer sequence (5′ – 3′)	Fluorochrome	Annealing temperature (°C)	[$MgCl_2$] (mM)
SB2[a]	F: ATCCATCTCTCTGTGCTC	TET™	50.0	1.5
	R: CAATTTGTTCCATGTTGATG			
SB7[a]	F: TAAGTGTCATAGAAGGAGAT	HEX™	50.0	2.0
	R: GGAGATATTCACCCTGTATT			
SB8[a]	F: AGAAACAAGCAGGAAATAAA	HEX™	50.0	2.5
	R: ATTACTTCAAACTAAAAAGC			
SB10[a]	F: TCTAAATATCACTTTGGCTG	FAM™	60.0	2.5
	R: TGGGCAACTTAGTAAGACC			
SB19[a]	F: GTGGTAGGAACAAGTAAAG	HEX™	47.5	1.5
	R: GTCAGGTGGGCTGTATG			
SB24[a]	F: ATCTGCCTATCACTTCTTTC	FAM™	56.0	1.5
	R: CATTTGCTCTGCTCATTCA			
SB30[a]	F: TAAAGTTAAGATTGGATTTCAC	FAM™	56.0	1.5
	R: GCAGAAAAACCTAACAATACA			
SB31[a]	F: TACCCGTACAGGATGCCAT	TET™	60.0	2.5
	R: GGTGCTAACGCTTTTGGTT			
SB37[a]	F: CACGAGAACACAAAGACAAA	TET™	60.0	1.5
	R: GAAAAACCTACCACCACTCT			
SB38[a]	F: GCCTCAATGGGTTTTAACC	PET®	60.0	1.5
	R: AGAACGAGTCTGTATCTTGA			
CJ7[b]	F1: TGTGCAAAGTCCCTGAAGTG[c]	NED™	63.0	1.5
	R: GGTTGCTATTTGCCAAGCAT			
CJ12[b]	F1: CAACCACAGATGCCAGCTT[c]	NED™	60.0	1.5
	R: TGATGGTGCATTCTTAGAGGG			

[a] Böhle and Zischler (2002)

[b] Nievergelt et al. (1998)

[c] Modified primer sequences

species 1, and backcross to species 2). We performed pairwise contrasts comparing *S. leucopus* to either *S. œdipus* or *S. geoffroyi*, including AMVA in the first analysis, and both CPN and CVC in the second one. We ran ten simulations of each dataset with Jeffrey's priors for 50,000 MCMC steps and a burn-in of 5000.

Mitochondrial Genetic Data

An effective way to identify the species affiliation of maternal lineages is the characterization of the mitochondrial genome, which is exclusively inherited from the mother in mammals. However, nuclear copies of the mitochondrial DNA, also known as numts, may share extensive sequence identity with authentic mitochondrial sequences,

and thus, be unintentionally amplified or coamplified (Soto-Calderón *et al.* 2012). To safeguard against amplification of numts, we amplified a fragment containing the mitochondrial hypervariable region I (HVI) using two different primer sets. First, we amplified a long fragment of *ca*. 4500 bp using the primers Sag-14652F (5′-CAAA GCCACCCTWACACGAT-3′) and Sag-2640R (5′-GCTCTGCCAYCTTAACAAGC-3′). As numts are usually below 500 bp in primates, this PCR product should be exclusively of mitochondrial origin (Bensasson *et al.* 2003; Mundy *et al.* 2000). Each 15 μl of PCR mix contained 20–50 ng DNA, 1× buffer (GC), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, and 0.2 U of *Taq* Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). Cycling conditions were as follows: 98 °C for 3 min, 35 cycles of 98 °C for 10 s, 67 °C for 30 s, and 72 °C for 3 min, with a final extension step of 72 °C for 10 min. We then sequenced both strands of the HVI with the primers Sag-15301F (5′-TACACCGGTCTTGTAAACC-3′) and Sag-16104R (5′-TCTGGCAAGACACAGTCAGG-3′), using standard Sanger methodology. We designed all mitochondrial primers from aligned mitochondrial sequences of New World primates available in Genbank.

We also amplified and sequenced a small fragment encompassing 700 bp DNA of the HVI, entirely contained within the long fragment described above, with the primer set Sag-15301F/Sag-16104R. PCR reactions consisted of 2050 ng of DNA, 1× KCl buffer, 2 mM $MgGl_2$, 0.2 mM dNTPs, 0.5 U of *Taq* polymerase (Fermentas), and 0.2 μM primers Sag-15301F/Sag-16104R in a final volume of 15 μl. PCR conditions were the following: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 1 min, and a final step of 72 °C for 10 min. Because sequencing of the *ca*. 4500-bp and 700-bp mitochondrial products yielded identical HVI sequences, we subsequently amplified and sequenced the small fragment from eight *Saguinus geoffroyi*, 20 *S. leucopus*, 13 *S. œdipus*, and the candidate hybrid specimens. In addition to the 41 sequences generated in this study, we retrieved 9 further HVI sequences from *S. œdipus* (KC757409) and *S. geoffroyi* (JN849588, JN849599, JN849618, JN849621, JN849622, JN849624, JN849632, and JN849633) from GenBank. The sequence of *S. œdipus* was originally obtained from a specimen belonging to a Primate Research Center in a non-native country (Finstermeier *et al.* 2013). These colonies were established several decades ago from wild-caught animals exported to foreign countries, before the adherence of Colombia to the Convention on International Trade in Endangered Species in 1981 (CITES 2017; Green 1976). The sequences of *S. geoffroyi* were generated from wild tamarins captured in the Panama Canal area by Díaz-Muñoz *et al.* (Díaz-Muñoz 2012). We edited and assembled DNA sequences with Geneious v6.1.5 (Biomatters Ltd.) and aligned them with ClustalW as implemented in MEGA7 (Tamura *et al.* 2007).

We used two methods to identify the affiliation of the maternal lineage of each hybrid with the three trans-Andean tamarins. First, we made a Bayesian phylogenetic reconstruction running a MCMC chain of 40 million steps with a 10% burn-in using the package Beauti/Beast v1.8.2 (Drummond and Rambaut 2007), adopting a HKY-G mutational model as selected by jModelTest 2 with the Bayesian inference criterion (Darriba *et al.* 2012). We then tested for convergence of all parameters in Tracer v1.6 (Drummond and Rambaut 2007). We generated a summary tree with TreeAnnotator v.1.8.2 and visualized it using FigTree v1.4.2 (Drummond and Rambaut 2007). To infer the relationships and mutational process connecting the mitochondrial lineages, we

made a network of HVI sequences (haplotypes) in Network v4.6.1.1 implementing the median-joining algorithm with default parameters (Fluxus Technology Ltd.).

Karyotypic Data

We identified chromosomal number and rearrangements in the parental species and hybrid specimens following a protocol of R-banding using 5-bromodeoxyuridine (BrdU) and Giemsa staining (RBG) (Camargo and Cervenka 1982). We initially established leukocyte cultures from 200 to 400 μl of heparinized blood of the four candidate hybrids, eight *Saguinus leucopus*, four *S. œdipus*, and three *S. geoffroyi*, following the protocol originally described by Moorhead *et al.* (1960). We cultured cells in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS, Gibco-Thermo Fisher Scientific) and antibiotics (100 μg/ml of streptomycin and 100 IU of penicillin [HyClone™, Chicago, IL, USA]) in a final volume of 7 ml. As mitogen, we added 70 μl of phytohemagglutinin working solution (PHA, Sigma-Aldrich) to the culture and incubated it at 37.5 °C for 60 h. We added 70 μl of 2 mg/ml BrdU (Sigma-Aldrich) to the culture 6 h before harvesting to induce R-replicative bands, and 70 μl of 10 μg/ml Colcemid (Gibco-Thermo Fisher Scientific) 1 h before harvesting to block cell division and obtain metaphasic chromosomes.

We harvested cells and obtained chromosomal preparations using standard protocols (Spowart 1994). We stained chromosomes according to Camargo and Cervenka (1982) with modifications introduced by López and Márquez (2002). We observed 80 metaphases per individual from two different cultures and captured digital images with a B/W camera (Basler, Ahrensburg, Germany) coupled to a Zeiss optical microscope (Oberkochen, Germany). We edited chromosome images using GIMP v2.8 (www.gimp.org) and assigned chromosome numbers according to their size.

Data Availability The microsatellite dataset analyzed in this study is available from the corresponding author on reasonable request. The mitochondrial sequences are accessible in the GenBank with Accession codes KT350488-KT350491, KT350494, and MH198231–MH198269.

Ethical Note

This research adhered to the legal requirements of the Colombian government. The National Authority of Environmental Licenses (ANLA) granted sample collection through permits #268 (February 1, 2013) and *Permiso Marco de Recolección* #0524 (May 27, 2014). The Colombian Ministry of Environment approved analysis of genetic data through the Non-commercial Access to Genetic Resources #87 (December 2, 2013). The Committee on Ethics and Animal Research at the University of Antioquia authorized animal handling protocols to I. D. Soto-Calderón (May 3, 2013). Our study followed the Guidelines of Best Practices for Field Primatology of the International Primatological Society. We have no conflicts of interest to declare.

Results

Fur Color Pattern of Hybrid Tamarins

The body proportions and measurements of the hybrids fell within the observed range in the three trans-Andean tamarin species (ESM Table SI). Similarly, body mass and body mass index fell within the range of the three trans-Andean tamarin species. The only observed exceptions were the two CPN twins, which were underweight (388 g and 370 g) and in poor physical condition. CPN facilities were envisioned as a temporary refuge (<180 days) for animals rescued from traffic. However, the large number of rescued animals, along with difficulties in rehabilitating and releasing animals into the wild, frequently results in longer stay periods and overpopulation, which translates into suboptimal conditions and limited nutritional resources and medical care.

Both CVC and the CPN twins had fur color patterns intermediate between *Saguinus leucopus* and *S. geoffroyi*, with white hands, black facial skin, and short white crest on the crown that turns red-brown toward the nape; and rump, feet, and proximal two-thirds of the tail covered in reddish or rusty red fur that turns black toward the tail tip (Fig. 1). They had a marginal fringe of white face hair and red-brown underparts, which are distinctive of *S. leucopus*, that contrasted with slightly squared ears and a yellow and black mottled back like *S. geoffroyi*, but reddish on flanks and shoulders. Unlike in CVC, white facial hair in CPN was sparse or absent toward the ears, with slight variation in the color pattern between twins.

In AMVA, the ventral side was rusty red and similar to that of *Saguinus leucopus*, and the crown and forearms were white like in *S. œdipus* but with shorter crown crest. The back, shoulders, and thighs were agouti gray-brown, the underparts red-brown, the forearms white, the feet yellowish white, and the tail reddish brown followed by a black section and a white tip.

Species Assignment and Inferred Ancestry from Nuclear Genetic Data

In cases in which we found genetic evidence of chimerism in blood samples, we successfully removed chimeric alleles through amplification from hair DNA. All loci were variable except for the locus CJ7, which was monomorphic in *Saguinus geoffroyi*. The mean number of observed alleles per locus varied from 4.5 in *S. geoffroyi* to 8.3 in *S. leucopus*. The effective number of alleles ranged from 1.0 to 5.1 in *S. geoffroyi*, 1.2 to 4.3 in *S. œdipus*, and 3.7 to 7.8 in *S. leucopus* (ESM Table SII). The observed heterozygosities varied from 0.56 in *S. œdipus* to 0.69 in *S. leucopus*. Allele frequencies were in Hardy–Weinberg equilibrium and only the loci SB19 in *S. œdipus* and CJ12 in *S. leucopus* showed significant heterozygosity deficit.

The Structure assignment test recovered three discrete clusters that match the parental tamarin species, with a few specimens of *Saguinus œdipus* showing shared ancestry with *S. geoffroyi*, as high as 27% (Fig. 2 and ESM Fig. S1). For CVC, 0.503 ± 0.028 of its genome was assigned to *S. geoffroyi* and 0.468 ± 0.027 to *S. leucopus*; for AMVA, the genetic components were 0.487 ± 0.001 *S. œdipus* and 0.508 ± 0.001 *S. leucopus*; for the CPN twins, the mean fraction of their genomes assigned to *S. geoffroyi* and *S. leucopus* were 0.502 ± 0.086 and 0.493 ± 0.085, respectively. Pairwise analyses with NewHybrids assigned all specimens in the three tamarin species to the correct parental class

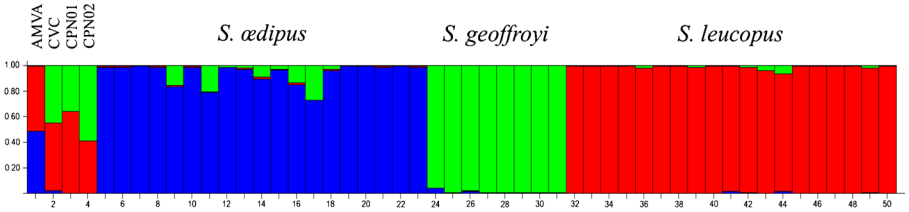

Fig. 2 Structure assignment test using 12 microsatellite loci to infer ancestry in tamarin hybrids. Values on the *y*-axis show ancestry coefficients, defined as the proportion of an individual's genome originated from one of the three previously delimited gene pools (red, green, and blue). Values on the x-axis represent the hybrids (1-4) and 46 reference specimens of *S. oedipus* (5-23), *S. geoffroyi* (24-31) and *S. leucopus* (32-50).

($P > 0.99$). Similarly, the analysis strongly assigned CPN and CVC to the *S. leucopus* × *S. geoffroyi* F1 class ($P > 0.91$), and identified AMVA as a *S. leucopus* × *S. œdipus* F1 hybrid ($P = 0.83$).

Identification of Maternal Lineage from HVI Sequences

The overall number of HVI haplotypes found in tamarins was 38, including 17 haplotypes in *Saguinus leucopus*, 13 in *S. geoffroyi*, and 8 in S. *œdipus*. Haplotypes

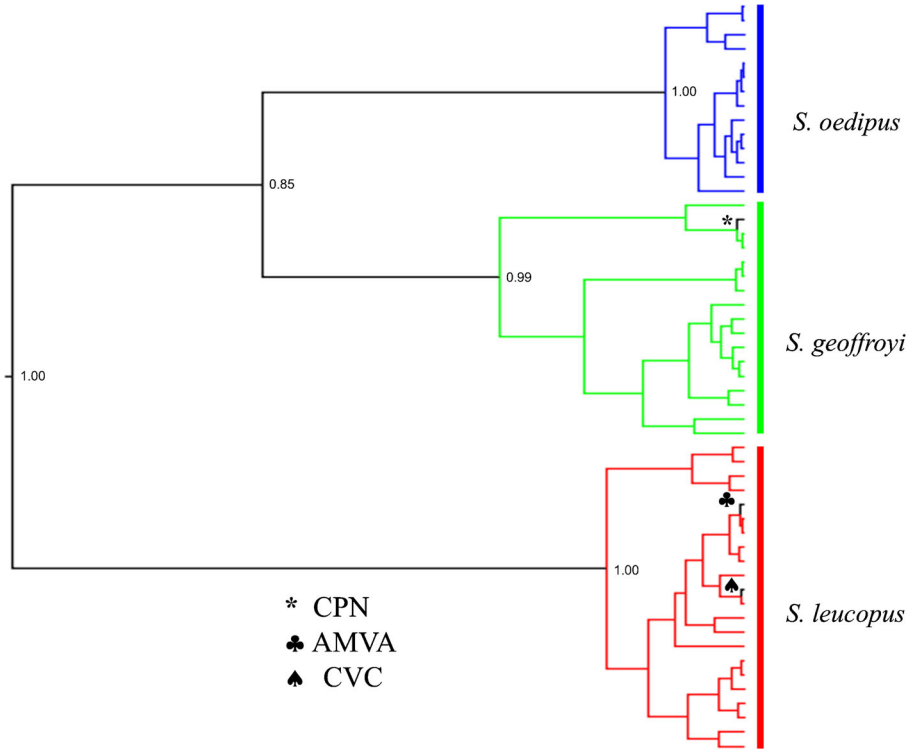

Fig. 3 Bayesian phylogenetic tree of trans-Andean tamarin species and hybrids based on sequences of the mitochondrial hypervariable region I. Mitochondrial lineages of *Saguinus leucopus*, *S. geoffroyi*, and *S. œdipus* are shown in red, green, and blue, respectively. Branch support is given by posterior probabilities shown in major nodes. Symbols (* - ♣ - ♠) indicate the mitochondrial lineage found in the hybrids.

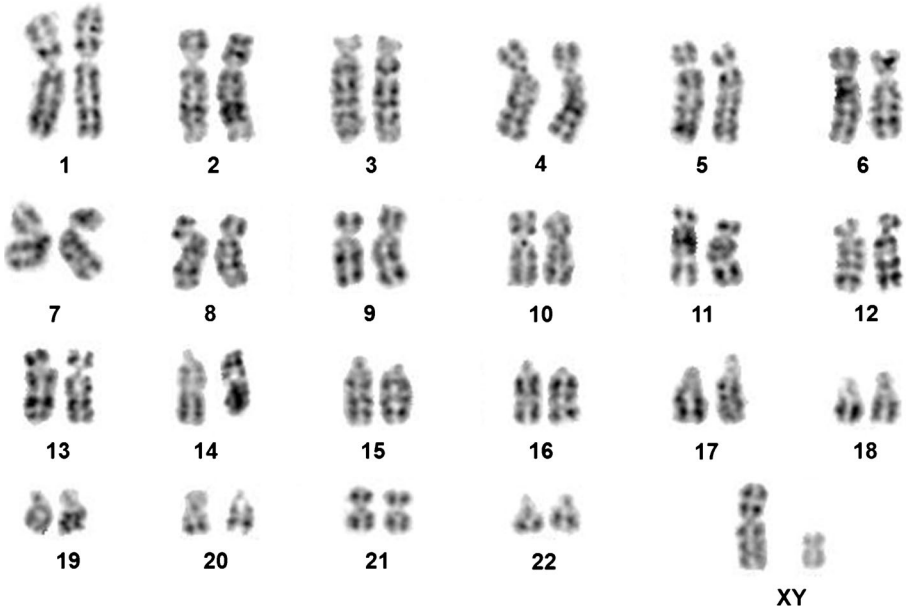

Fig. 4 R bands generated through incorporation of 5-bromodeoxyuridine (BrdU) and Giemsa staining in a hybrid tamarin (AMVA). Autosomal chromosome pairs were arranged according to their size (1–22).

were not shared among species and were well differentiated into three groups, as shown in the phylogenetic reconstruction and the network of mitochondrial sequences (Fig. 3 and ESM Fig. S2). The HVI sequences in AMVA and CVC were identical to others found in *S. leucopus*. The haplotype in CPN twins was also found in other *S. geoffroyi* individuals.

RBG Chromosomal Bands and Structure

The chromosome number ($2n = 46$) and the fundamental number (FN = 76) of chromosome pairs was conserved across species and hybrids. Surprisingly and despite exhibiting female genitalia, AMVA has a fixed XY karyotype with no apparent evidence of XX chimerism (Fig. 4).

Discussion

We describe three independent cases of hybridization between tamarin species in this study. They are, to the best of our knowledge, the first official reports of viable hybrids between recognized species of *Saguinus*. These hybridization events are supported by genealogical records in the case of CVC and the CPN twins and are consistent with morphological and genetic evidence. CVC and the CPN twins are documented first-generation hybrids of *S. leucopus* and *S. geoffroyi*, and coincidentally, they represent the offspring of reciprocal crosses between these two species. Inferences from two independent Bayesian analyses of nuclear genetic data allowed us to trace their origin

to the same two parental species in similar proportions, as expected for a first generation of genetic interbreeding. The maternal HVI lineages of CVC and the CPN twins were correctly assigned to the species of their known mothers (i.e., *S. leucopus* and *S. geoffroyi*, respectively). These data are consistent with first-generation hybridization events in both cases. The third case of hybridization is AMVA, a female of unknown origin whose fur color pattern hinted at a hybridization event between *S. œdipus* and *S. leucopus* that was supported by genetic data. The HVI sequence of AMVA was reliably assigned to *S. leucopus*. Inferences from microsatellite data also supported the hypothesis that AMVA is a first-generation hybrid between *S. œdipus* and *S. leucopus*, with similar proportions of these two species in the nuclear genome. Overall, these data indicate that AMVA is the offspring of a male *S. œdipus* and a female *S. leucopus*.

The AMVA's karyotype revealed a discordance between its chromosomal sex and the external appearance, consisting of a typical male XY karyotype but external female genitalia. Two underlying mechanisms may potentially explain this observation: cell chimerism in germinal cells and gonadal dysgenesis. In the first scenario, chimerism with an opposite-sex twin might affect the genetic constitution of the germ line and determine the sex of an embryo. As previously shown in a Wied's marmoset male (*Callithrix kuhlii*), chimeric cells from a male twin may become part of the gonads and the sperm cells (Ross *et al.* 2007). Whether chimerism between opposite-sex twins also influences the phenotypic sex in callitrichids is unknown, but if that were the case, an XY embryo with XX chimerism in the germ cells could develop female genitalia, as we observed in AMVA. We found molecular evidence of chimerism in one microsatellite locus (SB30) amplified from blood DNA of AMVA, but we failed to find XX metaphasic cells in the same sample. As blood and other hematopoietic tissues are particularly prone to harboring chimeric cells, the apparent lack of XX cells in blood suggests that 1) either chimerism occurred between two XY embryos or 2) XX chimeric cells are extremely scarce in blood. Unless XX chimeric cells are too rare to be detected in blood, opposite-sex cell chimerism might not explain the presence of female genitalia in a tamarin with male chromosomes as in AMVA (Benirschke and Brownhill 1962; Gengozian *et al.* 1964; Sweeney *et al.* 2012).

A more likely explanation to reconcile the chromosomal and genital sex of AMVA is gonadal dysgenesis (impaired development of male sexual structures). This is a cause of sterility in XY women who fail to grow functional male sexual structures during embryonic development due to mutations or an idiopathic dysfunction in the cascade that leads to testis formation (Iliopoulos *et al.* 2004; McCann-Crosby *et al.* 2014). Evidence from plant and animal genomes also shows that different patterns of DNA methylation and post-transcriptional gene silencing between species may result in altered levels of gene expression, activation of transposable elements, and genome instability in hybrids (Ha et al. 2008; Michalak 2009). Deleterious epigenetic effects on fetal growth and development have also been shown in hybrids of closely related mammal species (O'Neill *et al.* 1998; Zechner *et al.* 2004). Likewise, epigenetic differences between conspecific strains of *Drosophila* are responsible for impaired gonadal development in hybrids (Kidwell and Novy 1979; Michalak 2009). Whether differences in the epigenetic programming between species impacts the gonadal development of tamarin hybrids is still unclear. Future studies are needed to determine the relative role of hybridization and chimerism in the sexual determination of callitricids.

Beyond the putative gonadal dysgenesis that questions the fertility of hybrid AMVA, all the hybrid tamarins survived to adulthood with no external sign of disease or congenital defect. These observations demonstrate the presence of incomplete prezygotic reproductive barriers in *ex situ* tamarins held in animal shelters. However, whether interspecies hybridization occurs between populations in contact zones is unknown. *Saguinus leucopus* is geographically isolated from *S. geoffroyi*, but it is parapatric with *S. œdipus* and their geographical distributions are delimited by the Cauca River in the Colombian departments of Antioquia and Bolívar. Likewise, *S. œdipus* and *S. geoffroyi* are parapatric in the Urabá Gulf in northwest Colombia, and although their distributions are thought to be bounded by major rivers, the occurrence of potential contact zones remains to be determined (Defler 2010). Our Structure analysis revealed that several specimens of *S. œdipus* shared variable but minor levels of ancestry with *S. geoffroyi*. These two species diverged around 1.2 Ma and were considered as two subspecies of *S. œdipus* until the mid-1980s (Buckner *et al.* 2015; Hanihara and Natori 1987). The observed ancestry might be interpreted as shared ancestral polymorphism or as a consequence of genetic introgression from *S. geoffroyi* to *S. œdipus*. However, our NewHybrids analysis strongly assigned all *S. geoffroyi* and *S. œdipus* in our sample to two distinct species with no apparent trace of hybridization. The exact origin of the sampled specimens of these two species is unknown, and past hybridization might have occurred in captivity mediated by the illegal pet trade or in the wild in contact zones (Crispo *et al.* 2011; MADS 2012). Identification of contact zones is a necessary next step to determine the potential hybridization and introgression between trans-Andean tamarin species in the wild.

In contrast to wild populations of social primates, where visual, vocal, and olfactory cues, along with social behaviors, strengthen group cohesiveness and social structure, captive primates often develop idiosyncratic behaviors derived from isolation, confinement, and stress (Kappeler and Schaik 2002; Mendes *et al.* 2009; Olsson and Westlund 2007; Santana *et al.* 2012). This favors the emergence of circumstantial associations between specimens of different species that could lead to successful interbreeding, as in the tamarins in this study. Similar trends need to be explored in other heavily trafficked and taxonomically diverse Neotropical primates such as capuchins (*Cebus* spp.), marmosets (*Callithix* spp.), and howlers (*Alouatta* spp.), in which unintended hybridization is possible (Cortés-Ortiz *et al.* 2015). While this study provides evidence of hybridization and incomplete prezygotic reproductive barriers among species of tamarins (genus *Saguinus*), questions regarding the fertility of hybrids, genetic introgression in the wild, and the role of social bonds and other biological mechanisms in reproductive isolation in wild primates are important areas for future research.

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